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VOLUME 27 NUMBER 5

STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC AND HISTOCHEMISTRY

SEPTEMBER 1952

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OFFICIAL ORGAN OF THE BIOLOGICAL STAIN COMMISSION

Published by BIOTECH PUBLICATIONS, Geneva, New York

Entered as second class matter at the Post Office, Geneva, N. Y.

STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC AND HISTOCHEMISTRY

ISSUED BIMONTHLY

SUBSCRIPTION PRICE $5.50 PER YEAR (U. S. CURRENCY)

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Fast Green FCF	NGf–11	91%	Histology; cytology	June 27, 1952
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STAIN TECHNOLOGY

VOLUME 27 SEPTEMBER 1952 NUMBER 5

ETHYL VIOLET. A SELECTIVE DYE FOR THE ISOLATION OF GRAM-NEGATIVE BACTERIA

WARREN LITSKY, W. L. MALLMANN, and C. W. FIFIELD

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Received for publication Oct. 11, 1951

ABSTRACT.—**Minimal inocula of Gram-negative and positive bacteria were seeded into tryptose broth containing varying concentrations of dyes. Three dyes were used, namely crystal violet, brilliant green and ethyl violet. Growth rates were determined for 2, 4 and 6 hours incubation. All three dyes were equally effective in inhibiting Gram positive bacteria. Ethyl violet showed markedly less toxicity toward Gram negative bacteria than did either crystal violet or brilliant green.**

REVIEW OF LITERATURE

For many years advantage has been taken of the selective action of dyes inhibiting Gram-positive bacteria and allowing the growth of Gram negatives in the isolation of enteric bacteria. Drigalski and Conradi (1902) tested the selective action of malachite green, brilliant green, methylene blue, methyl violet and crystal violet. They prepared an agar medium containing crystal violet (1–100,000) and observed no inhibition of *Salmonella typhosa* but marked inhibition of cocci and other bacteria on this medium. Later, Conradi (1908) prepared a medium containing brilliant green (1–150,000) and picric acid (1–15,000) for the isolation of typhoid and paratyphoid bacteria. Since then many media have been prepared using the diamino triphenyl and triamino triphenyl methane dyes for the isolation of Gram-negative bacteria.

Churchman (1912) made the observation that Gram-negative bacteria were uninfluenced by a concentration of 1 to 100,000 of gentian violet. These observations were based on the growth response at the end of 24 hours incubation. This method of measuring the influence of inhibitory agents is commonly used today.

Revis (1911) observed that when a strain of *Escherichia coli* was

[1]Journal Article No. 1289 from Michigan Agricultural Experiment Station.

carried, by repeated transplanting, on a medium containing malachite green, gas production from lactose was inhibited. Later, he (1912) demonstrated on four strains of a coliform that, in training the organisms to grow in a 0.1% concentration of malachite green, gas production from lactose was permanently lost by two strains. He noted that the organisms showed a varying resistance to the dye.

Lommel (1926) confirmed Revis' studies. He trained coliforms to grow in the presence of malachite green (1–10,000) and safranin (1–4,000) respectively. By passage in malachite green, 9 out of 15 coliform organisms became inactive on lactose and in safranin 4 out of 16 coliform organisms were rendered inactive. Some of the strains permanently lost their ability to ferment lactose with gas production. No changes were induced in the fermentation of glucose, mannite, or maltose by any of the strains.

Darby and Mallmann (1939) demonstrated that brilliant green 2% bile broth exhibited a marked inhibitory action against *E. coli* as contrasted to rapid growth development in a 2% tryptose broth.

Experimental

The writers had observed that brilliant green (tetra-ethyl diamino triphenyl methane) was more bacteriostatic and more selective than was malachite green (tetramethyl diamino triphenyl methane). Ethyl violet bears a similar resemblance to crystal violet (hexamethyl triamino triphenyl methane) as far as structure is concerned.

A literature review failed to show any attempts to use ethyl violet in nutrient media. The dye has been used as a stain for spirochetes, the islets of Langerhans, and nerve tissue.

Tests were made to determine its inhibitory action on a selected group of bacteria using the technic of Mallmann and Darby (1939) for measuring growth during the first few hours of incubation starting with a minimal number of cells.

The base medium was tryptose broth prepared as recommended by Mallmann and Darby. Hundred-milliliter portions were prepared to which were added, prior to seeding, varied concentrations of each dye. The three dyes used were crystal violet, brilliant green and ethyl violet 6B. The first two dyes are certified by the Biological Stain Commission. The ethyl violet 6B was obtained from National Aniline & Chemical Co. (Lot No. 12552) containing 57.5% dye. The dye dilutions indicated in the tables refer to actual dye content.

The following Gram-negative bacteria were used: *Escherichia coli, Salmonella typhosa, Salmonella typhimurium* and *Salmonella pullorum.* The Gram-positive cultures were *Streptococcus faecalis* and *Bacillus subtilis.*

The seedings of each culture were between 8 and 19 organisms per ml. Duplicate plates were made on each sample at the time of seeding and after 2, 4 and 6 hours incubation at 35°C. All plates

were incubated 24 hours. Although all trials were repeated two to five times the tables show one representative trial.

In Table 1 are presented data on growth of *B. subtilis* in the presence of varying concentrations of the three dyes. It will be observed that even in a dilution of 1:10,000,000 both brilliant green and crystal violet are inhibitory although growth occurred in 6 hours. Ethyl violet was equal to brilliant green and crystal violet in inhibitory action at a concentration of 1:1,000,000. The data on *Strept. faecalis* were somewhat comparable in that a 1:1,000,000 dilution of all three dyes gave marked inhibitory results.

TABLE 1. THE GROWTH OF *B. subtilis* FROM MINIMAL NUMBERS AT 35° C. IN TRYPTOSE BROTH CONTAINING VARYING CONCENTRATIONS OF VARIOUS DYES

Hours of incubation	Number of Bacteria per ml.				
	Crystal Violet				
	None	1–10M	1–5M	1–1M	1–700
0	8	7	7	6	1
2	9	5	2	0	0
4	1,100	7	1	0	0
6	15,000	22	4	0	0
	Brilliant Green				
0	10	12	5	0*	0
2	17	1	2	0	0
4	21	1	0	0	0
6	14,000	0	0	0	0
	Ethyl Violet				
0	9	11	8	5	6
2	9	3	1	1	0
4	500	1	0	0	0
6	14,000	11	0	0	0

*The 0 counts obtained for *B. subtilis* in brilliant green at 0 hours incubation represent absence of growth rather than an absence of inoculum.

In Table 2 are presented data on the growth of *E. coli* in the presence of varying concentrations of the three dyes. Both crystal violet and brilliant green were markedly inhibitory at a concentration of 1:500,000 and 1:1,000,000 respectively. Brilliant green inhibited at all dilutions to a greater extent than did crystal violet. On the other hand, ethyl violet 6B was less inhibitory at a concentration of 1:200,000 than was either crystal violet at a concentration of 1:500,000 or brilliant green at a concentration of 1:1,000,000. The results obtained with *S. typhosa, S. typhimurium* and *S. pullorum* were comparable to those obtained with *E. coli*.

TABLE 2. THE GROWTH OF *E. coli* FROM MINIMAL NUMBERS AT 35° C. IN TRYPTOSE BROTH CONTAINING VARYING CONCENTRATION OF VARIOUS DYES

Hours of incubation	Number of Bacteria per ml.			
	Crystal Violet			
	None	1–1M	1–500T	1–300T
0	17	13	19	18
2	93	79	73	60
4	8,000	6,000	4,000	2,200
6	450,000	270,000	95,000	63,000
	Brilliant Green			
	None	1–1M	1–700T	1–500T
0	16	18	21	17
2	120	43	26	23
4	15,000	150	64	30
6	800,000	280	65	12
	Ethyl Violet			
	None	1–800T	1–400T	1–200T
0	19	18	21	22
2	140	117	115	114
4	15,000	9,500	9,500	9,500
6	700,000	700,000	450,000	300,000

CONCLUSIONS

Ethyl violet 6B (tetra-ethyl triamino triphenyl methane) in proper concentration inhibits Gram-positive bacteria.

Ethyl violet 6B shows less toxicity for Gram-negative bacteria than do crystal violet and brilliant green in comparable concentrations.

ACKNOWLEDGEMENTS

These studies were made possible through a grant from the National Institutes of Health.

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HISTOLOGICAL STAINING WITH METHYL-GREEN–PYRONIN[1]

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Received for publication Jan. 25, 1952

ABSTRACT.—**The standard technics for methyl green-pyronin staining are found to give inconstant results, often with poor differentiation between chromatin and cytoplasm. A modified procedure is described using n butyl alcohol for differentiation after aqueous methyl green staining and counter-staining with pyronin in acetone. After 6 minutes in 0.2% aqueous methyl green (chloroform extracted), the section is blotted, differentiated in n butanol, counterstained 30–90 seconds in acetone saturated with pyronin (less concentrated solutions may be preferred for some purposes), cleared in cedar oil and xylene and mounted. This technic retains the value of methyl green as a histochemical detector for polymerized desoxyribo-nucleic acid (DNA). The intensity of the stain, however, is considerably greater than that obtained with the procedure designed for quantitative (stoichiometric) photometric estimation of polymerized DNA. Pyronin serves primarily as a counterstain, and is not found to be a reliable indicator of ribonucleic acid either by this method or others which have been described.**

The use of methyl-green–pyronin, originally introduced as a differential stain by Pappenheim (1899) and modified by Unna (1902), has recently returned to favor as a histochemical stain for the differentiation of desoxyribose- and ribose-nucleic acids, here designated DNA and RNA, respectively (Brachet, 1940). The basis for this differential staining has been studied by us (Kurnick, 1947, 1950a; Kurnick and Foster, 1950; Kurnick and Mirsky, 1950; Devreux, Johanson and Errera, 1951). It was found that these two basic dyes, distinguished between the states of polymerization of nucleic acids and not between the acids themselves. Thus, highly polymerized nucleic acid (only DNA has been obtained as a sufficiently high polymer) stains with methyl green, while low polymers of both DNA and RNA stain with pyronin. For quantitative histochemical application, we considered pyronin unsuitable because of non-specific staining of protein and inconstant stoichiometry (Kurnick and Mirsky, 1950). A technic for the quantitative estimation of polymerized DNA was developed, based upon the photometric estimation of

[1]This work was assisted by grants from the National Heart Institute, U.S. Public Health Service, and the American Heart Association.

methyl green in homogeneous nuclei stained with an aqueous buffered solution (Kurnick, 1950b).

Methyl green, applied by the technic recommended for stoichiometric combination with polymerized DNA, yields too pale a stain, in all but the densest nuclei, for visual cytological study (as distinguished from photometric). Technics have been described for excess staining with methyl-green–pyronin mixtures, which are intended to preserve the qualitative differentiation between chromatin (green) and nucleoli and cytoplasm (pink). The most widely accepted of these (Cowdry, 1948; Lee, 1950; Conn, 1946) make use of Unna's mixture followed by rinsing in water, dehydration in acetone, clearing in cedar oil and xylene. In our hands this proved an unsatisfactory procedure, since the water rinse very quickly removed all the methyl green (including that from the nuclei) and the acetone removed the pyronin. These steps must therefore be performed with the greatest celerity, and at best the results are variable. The usual result is either a grossly understained section or one in which the nuclei are purple or red rather than blue or green. Adequate dehydration is difficult. Technics using ethyl alcohol rinsing for dehydration result in almost completely decolorized sections (cf. Rosa, 1950). Gerola and Vannini's (1948) use of buffered methyl-green–pyronin, followed by water and alcohol rinsing, does not escape the disadvantages of water and alcohol for differentiation.

Taft (1951) has introduced a modified procedure using a buffered aqueous methyl-green–pyronin mixture followed by rinsing in water and differentiation in a mixture of tertiary butyl alcohol and absolute alcohol. With this method we have occasionally obtained excellent results, but consistent results are, again, difficult to obtain. The disadvantage of rinsing in water is not eliminated: if only slightly prolonged, the nuclear stain is very pale, whereas if it is too brief, the cytoplasm is purple (the usual result with pyronin B according to Taft). The tertiary butyl alcohol mixture appears to remove the pyronin progressively (but less rapidly than acetone), and not altogether uniformly throughout the section, so that the intensity of the red stain, including that of nucleic-acid-free structures, such as mammalian erythrocytes, connective and elastic tissue, is a function of the duration of differentiation. Occasionally, if this step is too brief, purple nuclei, poorly differentiated from the cytoplasm are obtained. Exactly the same comments apply to Rosa's (1950) triethyl-phosphate–xylene differentiation.

In view of these difficulties, we have investigated alternative staining technics designed to give useful differential staining with methyl green and pyronin. The technics arrived at are not intended for quantitative application.

Materials

Human, rat and chick tissues fixed in cold acetone, cold 80%

alcohol, neutral formalin (4% formaldehyde), Carnoy's fluid, or Rossman's picro–formol–alcohol (Deane, et al, 1946), or by freezing in isopentane at −159°C. and dehydration *in vacuo* at −45°C., were embedded in paraffin at 54°C. After sectioning these were deparaffined in xylene and brought to water through graded alcohol-water mixtures. No technic yielded green chromatin after Rossman's fixation, presumably because of depolymerization of the DNA by the picric acid (Kurnick, 1950a). It is therefore omitted in the general discussion which follows.

The dyes used were (a) pyronin B, National Aniline Div., C.I. No. 741, Cert. No. NP 11,[2] and (b) methyl green, National Aniline Div., C.I. No. 685, Cert. No. NG 26. The latter was dissolved in water (1% commercial dye solution) for the Unna stain mixture and in 0.2 *M* pH 4.2 acetate buffer (0.4% crude dye) for the buffered stain. Both solutions were repeatedly extracted with chloroform until the chloroform layer was colorless (to assure removal of crystal violet) (Kurnick, 1950a). Taft (1951) has confirmed our observation that more complete extraction is possible from solution than from the dry state. Unlike Taft, we have found the $CHCl_3$-extracted buffered methyl green solution to be stable for 3 months or longer, as determined by the absence of $CHCl_3$ soluble dye.

Unna's mixture was prepared according to Cowdry (1948), slightly modified to permit the use of $CHCl_3$-extracted methyl green solution: 1.0 g. pyronin B dissolved in a mixture of 2.5 ml. alcohol, 20 ml. glycerol, 22.5 ml. 1.0% aqueous phenol, plus 50 ml. 1% aqueous methyl green.

The buffered stain was prepared by mixing 1 vol. 0.25% aqueous pyronin with 1 vol. 0.4% buffered methyl green.

All reagents used were C.P. grade.

Methods and Results

A. Unna's mixture

The results of using water-acetone differentiation were referred to in the introduction. These were obtained with all the fixations used except Rossman's, which yielded red to pink nuclei only, never purple, blue or green. The length of time of immersion in the stain mixture was varied from 6 minutes to 1 hour without significant effect on the result, which appeared to be influenced only by the length of time of rinsing in water or acetone.

If the section is observed after rinsing in water alone, the entire tissue, including nuclei, is red. Hasty rinsing preceded by blotting, and more leisurely rinsing yield the same result at this point. Rinsing in acetone (at the next step) removes pyronin. The intensity of the pink color depends upon the rapidity of acetone dehydration.

[2]Some packages of this stain batch (NP–11) were labeled Pyronin Y, C. I. #739. Dr. Conn has advised us that Pyronin Y is no longer available, and that batch NP–11 and all subsequent batches are, in fact, pyronin B.

It is now found that if the water rinsing has been exceedingly brief, the nuclei will vary from green to purple (except after Rossman's fixation), depending upon the amount of pyronin left in the section; if the water rinse has been less rapid, the nuclei vary from very pale brownish pink to red, generally somewhat more intense than the surrounding cytoplasm.

If rinsing in acetone is performed rapidly in Coplin jars, it is often noted that the top section on the slide is pink, the bottom section contains no pyronin, and the sections between show a progressive reduction in the intensity of the pyronin stain from top to bottom.

If the water rinse is omitted and acetone used directly (after blotting excess stain from the section), only pyronin appears to be removed so that the entire section becomes at first purple, then blue, then green. There is poor differentiation between nuclei and cytoplasm since the latter retains varying amounts of green depending upon fixation (most after Carnoy). If the section is now rinsed in water, methyl green is removed so that the end-result resembles that described in the preceding paragraph.

Variations of only a few seconds in the rinsing steps determine the character of the result. Thus, only rarely is a satisfactory stain obtained, and the results are not reproducible.

Since water was capable of removing almost all the methyl green from both cytoplasm and nuclei without removing the pyronin from the nuclei, and acetone removed all the pyronin without completely removing the methyl green from the cytoplasm, it appeared desirable to avoid both of these solvents after the Unna stain mixture, just as ethyl alcohol had been discarded in the past. Accordingly, a search for other agents was made.

Tertiary butyl alcohol and iso-propyl alcohol were found to behave like acetone, leaving both cytoplasm and nuclei green. Normal butyl alcohol removed all the pyronin from the entire section, but removed methyl green differentially: only chromatin and cartilage matrix retained the stain. The chromatin remained brilliantly green even after prolonged differentiation in several changes of *n* butyl alcohol. Iso-amyl alcohol failed to differentiate sections uniformly, probably because of its poor miscibility with water. It tended to give green nuclei and pale purple cytoplasm. Very prolonged application produced results similar to tertiary butyl alcohol. A mixture of 4 parts tertiary butyl alcohol and 1 part water completely decolorized the slide.

Since *n* butyl alcohol gave excellent differentiation of the methyl green, while removing all the pyronin, *n* butyl alcohol saturated with pyronin was tried for rinsing the slides after staining. It was found that pyronin B was less soluble than 1 part in 1000 *n* butyl alcohol (although a saturated solution is deep red), while purified methyl green is practically insoluble in *n* butyl alcohol. Normal

butyl alcohol saturated with pyronin gave results indistinguishable from those with *n* butyl alcohol alone, in that no pyronin remained in the sections.

Having obtained sections well differentiated in regard to methyl green by *n* butanol rinsing, it remained necessary to reintroduce the pyronin without removing the methyl green from the chromatin. It was found possible to do this by immersing the slide for ½ to 2 minutes in acetone saturated with pyronin (it is important that this solution be free of water; fresh solutions are, therefore, preferable). This also had the advantage of maintaining the dehydration of the tissue, permitting clearing in cedar oil directly from the acetone-pyronin. The result was bright clear green chromatin, red cytoplasm and pink nucleoli (often obscured in low power observation by the nucleolar-associated chromatin which was intensely green).

B. BUFFERED AQUEOUS STAIN

The results with this mixture were in all respects similar to those obtained with the Unna mixture except that fairly satisfactory results were obtainable with acetone rinsing alone. After 5 to 30 minutes in the stain mixture (the longer times had no notable advantages), slides were blotted, rapidly rinsed in acetone, and transferred to cedar oil. The methyl green was completely removed from the cytoplasm. The degree of pyronin staining was again a function of the length of time of immersion in acetone, so that with brief rinsing, cytoplasm was red and nuclei purple, whereas with longer immersion, cytoplasm was pale pink to colorless and the chromatin blue to green. The satisfactory differentiation of the green from the cytoplasm by acetone alone, as contrasted to the result after staining in the Unna mixture, appears to be a function of the lower methyl green concentration in this mixture, since if the concentration is increased to 0.5% (as in the Unna mixture and that of Taft, 1951), the cytoplasm retains a green tint if the water rinse is omitted.

If Taft's methyl-green–pyronin procedure is followed with omission of the water rinse (simply blotting) before differentiation in the tertiary-butyl-alcohol:ethyl-alcohol mixture, pale green to purple cytoplasm and green to bluish-purple nuclei result. Since the removal of pyronin is slower than with acetone, better control is possible. The results are similar to those obtained with the complete procedure as described by Taft, but with more densely stained chromatin.

The triethyl-phosphate–xylene mixture of Rosa (1950), used after Taft's stain with omission of the water rinse, gives results like those with tertiary butyl alcohol and ethyl alcohol.

With the other alcohols as differentiating media, the results were identical to those obtained with the Unna mixture.

C. MODIFIED STAINING METHOD

Since the best differentiation was obtained with *n* butyl alcohol, which removes all the pyronin from the section, it was considered unnecessary to include pyronin in the first staining step. Accordingly, we attempted staining 6 minutes with 0.2% methyl green in 0.1 *M* pH 4.2 acetate buffer or in water (both exhaustively extracted with $CHCl_3$ before use) followed by blotting and immersion for several minutes in two changes of *n* butyl alcohol. The slide was

FIG. 1. — Human liver biopsy, Carnoy fixation, stained with methyl green and pyronin, as in C, p. 237; 4 mm. (40×) apochromatic objective, 12× periplan eyepiece, photographed at 36 cm. ocular to plate distance, on Kodak 50 plate. Enlarged 2.5× in printing.

then immersed in acetone saturated with pyronin for 30 to 90 seconds depending upon the intensity of red desired in the cytoplasm; dilute acetone solutions of pyronin (10% saturated) may be used if more delicate staining is desired. The slide was transferred directly to cedar oil, then xylene and mounted in Permount.

This technic yielded red cytoplasm, pink nucleoli, bright green chromatin, brown erythrocytes, green cartilage matrix, pink (with a trace of violet) osseous matrix.

D. EFFECT OF NUCLEASES.

1. Desoxyribonuclease. Sections of rat liver and kidney and human liver fixed in 80% alcohol or Carnoy's were immersed for 2 hours at 37° C. or 24 hours at 21°C. in 0.02 mg./ml. desoxyribonuclease (DNase)[3] in 0.01 *M* trimethylol amino-methane buffer (Gomori, 1946) at pH 7.6. Two adjacent sections served as controls. One was brought to 50% alcohol from xylene and left there until the others had been treated, then brought to water and stained. The second was deparaffined, brought to water and exposed to the buffer without the nuclease. The slides were then rinsed in H_2O, 70% alcohol, H_2O in that order, and stained as in C. Both control slides revealed, as usual, green nuclei and red cytoplasm. In the nuclease-treated sections, the cytoplasm and nucleoli stained red, but the chromatin was colorless or revealed a faint trace of pink. The sections were decolorized by immersion in acetone, water, 50% alcohol, water in that order. They were then stained 3 minutes in 1% toluidine blue[4] in 95% alcohol, rinsed in water, dehydrated in acetone, cleared in xylene and mounted. The DNase-treated slide was practically unstained except for a trace of blue in some nuclei. The untreated control revealed deep blue nuclei and pale blue hepatic cytoplasm, while renal cytoplasm was practically unstained. The buffer treated control showed deep blue nuclei, but no cytoplasmic staining.

2. Ribonuclease. Sections of rat liver and kidney were immersed for 2 hours at 55°C. or 24 hours at 21°C. in 1 mg./ml. ribonuclease (RNase),[5] in 0.05 *M* trimethylol aminomethane buffer at pH 7.6. Two controls were also prepared as in D1. Staining was carried out as in C. Both the control and the RNase treated sections stained as usual with red cytoplasm and green nuclei. The procedure was repeated, modified slightly by rinsing in acetone after staining in acetone saturated with pyronin. The result was only to reduce the intensity of pyronin staining in all sections. If one-tenth saturated pyronin in acetone for 3 minutes was substituted for the saturated acetone, the result was a much less intensely stained, but still pink cytoplasm in all sections. The standard methyl-green–pyronin procedure (water and acetone differentiation after 6 minutes in Unna's mixture) and Taft's method also failed to reveal any constant distinction between either control section (untreated and buffer treated) and the RNase treated sections. When such sections were decolorized and stained with toluidine blue (see D1 above), the buffer treated and RNase-treated sections revealed deep blue nuclei and colorless cytoplasm, while the section which was exposed to neither had deep blue nuclei and pale blue cytoplasm in the liver slices (kidney cytoplasm did not stain).

[3]Purchased from Worthington Laboratories, Freehold, N. J.
[4]Toluidine blue O, Harleco, Color Index No. 925, Cert. No. NU–8.
[5]Purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.

Discussion

The usual differentiation after methyl-green–pyronin staining in water is found to remove the color from the nuclei too readily, while the acetone step removes the pyronin. The substitution of tertiary butyl alcohol (Taft, 1951) or ethyl phosphate (Rosa, 1950) for acetone permits better control of the pyronin differentiation, since they are slower, although somewhat irregular. These media do not differentiate the methyl green, which depends upon the unsatisfactory water rinse. However, if the methyl green concentration in the stain mixture is reduced to 0.2%, the water rinse may be omitted with fairly satisfactory result and only slight cytoplasmic staining with methyl green (so that the cytoplasm appears purple rather than red).

We have found that *n*-butyl-alcohol differentiation and pyronin-in-acetone counterstaining give very good reproducible differentiation of cytoplasm, nucleoli and nuclei. The modified procedure appears to retain the specificity, in animal tissues, of methyl green for polymerized DNA (except for its staining of cartilage), as indicated by the fact that strong acid fixatives and DNase destroy the affinity of chromatin for methyl green. The former, which depolymerize but do not remove the DNA (as confirmed by the affinity of the chromatin for toluidine blue), make the chromatin pyroninophilic (Kurnick, 1950a), whereas the DNase which removes the DNA (note loss of affinity for toluidine blue) renders the chromatin essentially unstainable by the methyl-green–pyronin method proposed by us. However, the procedure is not thought to provide stoichiometric staining with methyl green, as is accomplished with aqueous buffer washing, since the *n*-butyl-alcohol technic provides considerably more intense staining than the aqueous procedure (Kurnick,1950b). The results with the latter yield the same stoichiometry as is found in the test tube (13 P atoms per dye molecule), but the nuclei are too pale for visual cytological examination. Whether the *n*-butyl-alcohol differentiation gives constant staining, suitable for quantitative interpretation remains to be determined by photometry of homogeneous nuclei (see Kurnick, 1950b). It can be said, however, as would be expected in view of the constancy of DNA per diploid nucleus of an organism (Boivin, Vendrely and Vendrely, 1948: Kurnick and Mirsky, 1948; Mirsky and Ris, 1949), that large nuclei (such as liver nuclei) stain less intensely than small nuclei (such as fibroblasts and leucocytes), suggesting at least a semi-quantitative application of methyl green in the staining procedure described.

In the technic we have recommended for histological staining, pyronin serves only as a counterstain. The removal of ribonucleic acid by ribonuclease or by 0.01 to 0.05 *M* trimethylol amino-methane buffer (pH 7.6) failed to influence pyroninophilia when pyronin in acetone was used for staining or when any of the standard methods

were applied by us. This is contrary to the experience of others (Brachet, 1940), and may be a function of the stain used. We have used a pyronin B certified for use in the Unna-Pappenheim procedure; but this may be less satisfactory than the no longer available Pyronin Y (cf. Taft, 1951). Our earlier observation on slight protein staining with pyronin *in vitro* (Kurnick, 1950a) is in accord with the staining of sections found here. Since we have not found pyronin capable of serving as an indicator for RNA by the usual staining procedure or by any modification we have attempted, we prefer other basic stains such as toluidine blue (or ultraviolet miscroscopy) for histochemical analysis of ribonucleic acid.

It may be worthy of note that in the staining procedure described, perinuclear pyroninophilic granules are found in some normal human liver aspiration biopsies (fixed in Carnoy's fluid) (Fig. 1.). (Cf. McKay and Farrar, 1950.) These granules resemble those seen in the livers of well fed rodents. Rich and Berthrong (1949) have described similar inclusions in post-mortem human material from patients dead of infectious diseases and Pollister *et al* (1951) have noted them in livers from a large variety of human autopsy material, but never in liver biopsies. Our findings suggest that the failure of other observers to find these granules in biopsy material may have been due to their fasting of the patient prior to surgical intervention. It has long been known that in starvation (Berg, 1914), rodent liver cells very rapidly lose their basophilic cytoplasmic granules. The same probably occurs in human material. It is proposed to investigate this point directly.

We have observed no advantages in the use of glycerol, alcohol, and phenol in the stain (cf. Taft, 1951). When added to mixtures of methyl green and pyronin or to either dye alone, we have observed no modification of the result obtainable with simple aqueous solutions as to specificity, intensity, or quality of stain. We have been unable to detect any rational basis for their incorporation in the stain either *in vitro* (Kurnick, 1950a) or in histological staining, and have therefore omitted these reagents from the stain in our method. The adjustment of the pH to the range in which the color of methyl green is stable (pH 3–5) is more reliably accomplished with buffers than with phenol.

It should be emphasized that if formalin is used as a fixative, it must be accurately adjusted to pH-7.0. Fixation must be brief, lest the solution become acid (marble chips do not sufficiently stabilize the pH). If these precautions are not taken, faint green staining of cytoplasm may be noted in sections treated by the method. This problem does not arise with the other fixatives.

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PREPARATION AND TESTING OF SILVER-PROTEIN COMPOUNDS

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Received for publication April 17, 1952

Abstract.—**Silver derivatives of the protargol type were made from 13 different commercial peptones. The peptone samples were purified by precipitating with an ethanol concentration of 75%, reprecipitating once, and discarding any water-insoluble material. A 20–22% solution of the purified material in water, alkalinized with 2 ml. of strong ammonia per 100 ml., was precipitated by adding an equal volume of 25% aqueous $AgNO_3$, and stood in a refrigerator overnight. Thorough washing of the precipitate with distilled water, draining, and then dissolving it in a purified peptone solution (30–35% aqueous), whose volume was 0.6 that of the solution used for silver precipitation, gave the soluble silver derivative. Ammonia was added to facilitate solution and the final pH adjusted to 8.0–8.4. The concentrated solution was dehydrated either with acetone or in a dessicator under reduced pressure and ground to a powder. Staining tests on neurological tissue showed that the *Pharmaceutical peptone* (Cudahy Packing Co., Omaha, Nebr.) and the *Bacteriologic peptone* (Wilson Laboratories, Chicago, Ill.) gave the best preparations for staining nerve fibers, and that these were essentially equal to the formerly available German made *Protargol*.**

In an earlier paper (Porter and Davenport, 1951), a brief history of the adoption of silver proteinates for neurological staining was given and the results of some experiments in the preparation of this type of compound were discussed. Another discussion of silver-protein compounds and the factors which influence their behavior as neurologic stains has been published by Romanes (1950).

Pharmaceutically, such compounds are classified as strong protein silver and contain about 8% Ag. Only one brand, Protargol (Winthrop), particularly the prewar material of German manufacture, possessed superior properties as a neurological stain. Other brands, although pharmaceutically quite satisfactory, have exhibited little or no staining affinity for neural structures.

We are concerned presently with the silver derivatives prepared from some of the commercially available protein hydrolysates. Such hydrolysates are made from various proteins and differ in degree of cleavage of the original protein and also means of cleavage (acid or

[1]Publication No. 586.

enzyme). In order to compare samples from different manufacturers, a standard technic for the preparation of the silver derivative was adopted. The technic is an arbitrary one but is based on observations that the portion of a protein hydrolysate insoluble in 70–75% ethyl alcohol yet freely soluble in water gave the most active staining compounds. In describing the procedure, the term *peptone* will be used to denote the original commercial hydrolysate and *purified peptone* the water-soluble fraction precipitated by 75% alcohol. *Water* refers to distilled water.

Procedure

1. Forty grams of peptone (dry powder) were suspended in 150 ml. of absolute alcohol in a 500 ml. Erlenmeyer flask, and while shaking the mixture, 50 ml. of water added. Much of the material aggregated and was allowed to settle. The supernatant was discarded.

2. The precipitate was dissolved in 40–60 ml. of water (volume not critical) at 60–70°C., cooled to room temperature, and 3 volumes of absolute alcohol added slowly while shaking the solution. About an hour was allowed for settling and the alcohol-water mixture decanted as completely as possible and discarded.

3. The residue was dissolved in 40 ml. of water at 60–70°C., cooled, and 1.5 ml. of strong ammonia water (sp. gr. 0.9) added. At this stage, insoluble material was usually removed by centrifugation, and the solution divided into two equal portions. To one portion (designated A) 15 ml. of water was added. The undiluted one was designated B.

4. Ten grams of C.P. or reagent $AgNO_3$ were dissolved in 35 ml. of water and this solution added slowly to portion A of the purified peptone solution. The latter was shaken vigorously during the addition and the preparation set aside in the dark 3–5 hr. at 25°C. or 16–20 hr. at 5°.

5. The precipitate usually settled well and adhered to the flask. The supernatant was decanted and discarded and the precipitate washed with 3 changes of water of about 200 ml. each, with 0.5 to 1.0 hour intervals between changes. When the precipitate had adhered as a gummy mass to the bottom of the flask, it was broken up to facilitate washing.

6. Portion B of the purified peptone solution was added to the washed and well drained precipitate. Solution of the latter was facilitated by warming to 60°C., rubbing it against the side of the flask with a glass rod, and the drop by drop addition of strong ammonia water. The pH was tested colorimetrically by placing a few drops of the solution in a test tube, diluting with water to 10 ml., and adding 2–3 drops of phenol red indicator solution (0.05% dye concentration). A color near the alkaline end of the indicator's range between pH 7.8 and 8.4 usually coincided with the complete solution of the precipitate. However, all preparations were adjusted

to a pH within this range to provide uniformity for subsequent staining tests.

At this stage of the preparation, the concentrated solution may be diluted at 500–1000 ml. for use as a staining solution, but if the material is to be stored, it should be reduced to a dry powder.

7. When the precipitate had dissolved completely, the solution was transferred to a glass mortar of about 150 ml. capacity and 75 ml. of anhydrous acetone poured on top of it. The mixture was then stirred rather slowly with a glass rod until the silver peptone solution became viscous. The addition of too much acetone accompanied by vigorous stirring favored the formation of a colloidal suspension instead of effecting simple dehydration by the acetone. As dehydration proceeded, the acetone was poured off from time to time and fresh added. After several changes, accompanied by working the mass with a pestle, it became brittle enough to grind and was reduced to a powder under acetone. The resulting acetone suspension was either filtered through paper, centrifuged, or simply allowed to settle, and the acetone discarded. The acetone-wet material was then spread on a glass plate and stirred constantly until dry. The stirring was necessary to prevent rehydration by condensed moisture from the air.

The technic requires no elaborate apparatus and can be carried through without a centrifuge; however, this machine can be used advantageously when available. Drying the wet product (step 7) in a vacuum desiccator, with subsequent grinding, can be employed to avoid the rather tedious dehydration with acetone. Dehydration should be thorough because the pulverized material will form a gummy mass in storage if all the water is not removed either before or during grinding.

The best consistent yields of silver derivative were obtained with Cudahy's *Pharmaceutical peptone* and this yield was about 10 g. of dry material from the quantities given in the technic.

Commercial Split-protein Products Tested

1. Proteose peptone, Type 2. Armour Laboratories, Chicago, Ill.
2. Phytone. Baltimore Biological Laboratory, Baltimore, Md.
3. Protone. 4. Bacto-peptone. Difco Laboratories, Inc., Detroit, Mich.
5. Pharmaceutical peptone. Cudahy Packing Co., Omaha, Nebr.
6. Lactalbumin hydrolysate (enzymatic). Nutritional Biochemical Corp., Cleveland, Ohio.
7. Casein hydrolysate. E. R. Squibb & Sons, New York, N. Y.
8. B Hydrolysate. 9. Brewers' peptone. 10. R peptone.
11. Bacteriologic peptone. 12. Thio-peptone. 13. Medo-peptone. The Wilson Laboratories, Chicago, Ill.

Not all of these materials went through the process for making their silver derivatives smoothly. Only numbers 5, 7, 8, 9 and 10

formed a clear solution when the precipitate from step 1 was dissolved as specified in step 2. The R peptone (No. 10) formed a colloidal suspension when the silver nitrate solution was added (step 4) and only a few milligrams of its silver derivative were recovered. The lactalbumin hydrolysate (No. 6) precipitated well, but the silver precipitate redissolved (step 6) only partially. Difco protone (No. 3) was refractory both toward making a concentrated aqueous solution and the resolution of the silver precipitate.

Some water-insoluble material (in the high concentrations used) was encountered in samples 1, 11, 12 and 13. In some preparations, this undissolved fraction was removed by centrifugation, while in others it was not. Subsequent staining tests indicated that the turbid staining solution, which resulted from allowing the insoluble fraction to go through the preparation, sometimes caused precipitates on the slide but did not otherwise affect the staining reaction.

Yields of the silver compound varied rather widely among the different samples. Starting with 20 g. of a split protein and adding 10 g. of silver nitrate to the fraction that came through the alcoholic precipitation, yields of dried powder (pharmaceutically, strong silver protein) varied from 2 to 10 g. in all but the most unsuccessful preparations. About 25 of these finished products were analyzed for silver

PLATE 1

FIG. 1. — Sciatic nerve; cross section. (a). Stained with a silver compound made directly from Cudahy pharmaceutical peptone (not precipitated by alcohol). (b). Peptone purified by precipitation before combining it with silver. Strengthening of the staining of axis cylinders is shown by comparison with (a).

FIG. 2. — Cerebellum stained with old protargol (German) No. 4948.

FIG. 3. — A section adjacent to the one shown in 2 stained with one of our silver preparations (Batch 20A) made from purified pharmaceutical peptone. (a). Subpial part of the molecular layer in which the terminals of the Purkinje cell dendrites appear as branching filaments. (b). The minute, very numerous black dots above the Purkinje cells are cross sections of fine nerve fibers.

FIG. 4. — Cerebellum, but the direction of sectioning is at a right angle to that of the preparations shown in 2 and 3. The Purkinje cells are edgewise to the plane of the section, hence the fine fibers seen in cross section in 2 and 3 are longitudinal in 4. Stained with a silver derivative of Wilson's bacteriologic peptone purified by precipitation.

FIG. 5. — Cerebrum. The pia mater lies at the top of the figure and the "subpial plexus" of nerve fibers is just below it. Stain: Batch 27 from Cudahy peptone.

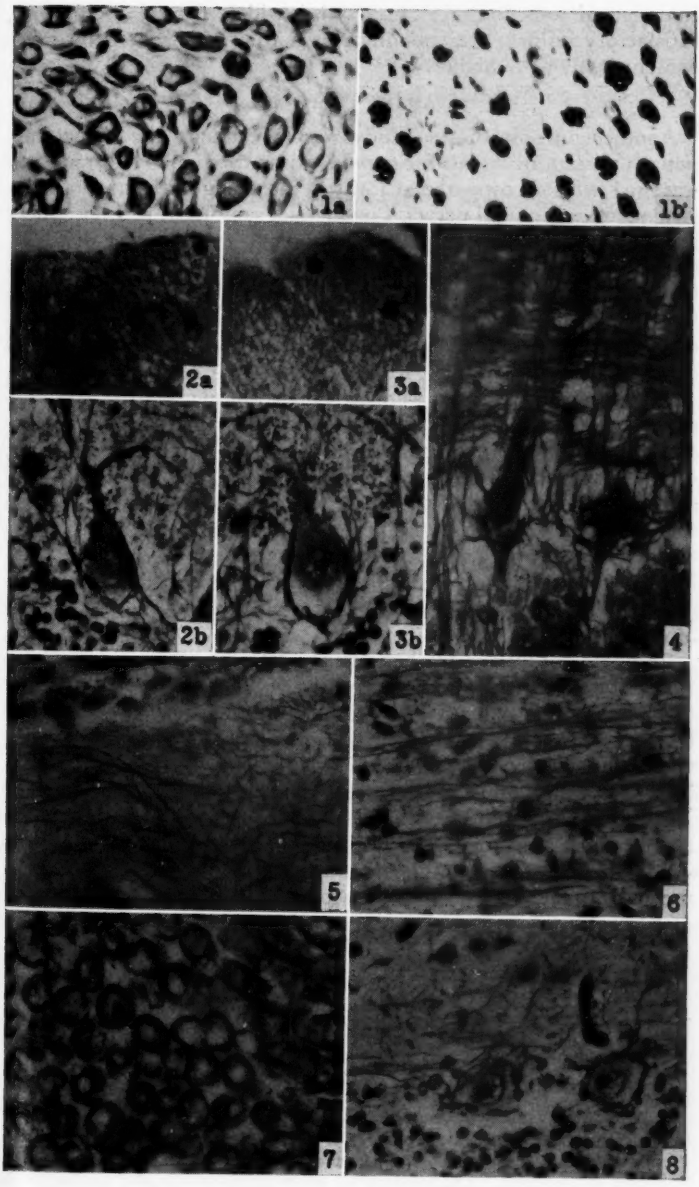
FIG. 6. — Cerebrum; fetal. Parallel fascicles of developing nerve fibers are shown. Same stain as 5. (Old protargol failed on this one).

FIG. 7. — Sciatic nerve, cross section. Stained with the silver compound made from lactalbumin hydrolysate. Myelin sheaths are conspicuous.

FIG. 8. — Cerebellum. Stained with the silver derivative of Wilson's Medo-peptone. Cell nuclei predominate but some of the coarser nerve fibers are stained.

Magnifications: Fig. 1, 1000; all others 500. All tissues except 1a and 1b, cat; 1a and 1b, monkey. All are from adult animals except 6, which is from a fetus near term.

Fixations: No. 6, 10% formalin; all others Bouin's fluid.


1a
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2a
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content and were found to vary rather widely from 4 to 10% Ag. Satisfactory staining was obtained, as a rule, only when the silver content was 7% or higher.

Staining Properties

Another purpose of this study was to evaluate the silver compounds as staining agents for nervous tissue. Bodian's (1936) method was used (without copper) on cat tissue, including the central nervous system and peripheral nerves. Commercial samples 1, 5, 9, 11 and 12 gave silver derivatives that were definite axis cylinder and dendritic stains. Derivatives of 2 and 6 had a predilection for myelin sheaths and that of 13 stained cell nuclei rather selectively. The others varied from having only a slight affinity for tissue to being general cellular and connective tissue stains. Figures 1–8 illustrate some of the staining reactions.

The two best peptones were Cudahy's pharmaceutical and Wilson's bacteriologic. The Cudahy material was more freely soluble and gave somewhat higher yields of silver derivative than Wilson's, but the product made from either gave good neural staining reactions. The finding that the pharmaceutical peptone is well suited for the preparation of strong protein silver agrees with that of the Sterling-Winthrop Research Institute (personal communication from Dr. M. L. Tainter, Director). This peptone, in conjunction with gelatine, has been used in the preparation of *Protargol* (Winthrop-Stearns, Inc.).

Comparisons were made between the staining reactions of a number of our present preparations and the old (German) *Protargol,* Lot No. 4849. There appears to be little or no difference between our best lots and the old material. Either of the two peptones mentioned above when purified by precipitation with alcohol will serve for making silver compounds which give good neurological staining. The preparations that have been made without the preliminary purification of the commercial products have been rather erratic in their staining behavior, hence the separation of the fraction to be used by precipitating it with 75% alcohol seems to be justified.

Acknowledgements

The cost of this research was supported largely by a grant from the Biological Stain Commission. Peptone samples from the Wilson Laboratories and Armour Laboratories were furnished gratis.

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A RAPID METHOD FOR MOUNTING POLLEN GRAINS[1]

WITH SPECIAL REGARD TO STERILITY STUDIES

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Received for publication March 31, 1951

ABSTRACT.—**A method is described for the simultaneous mounting and double staining of mature pollen grains. The medium consists of 50 ml. of glycerol jelly to which 2.5 ml. of methyl green and 2 ml. of phloxine, both in 50% alcoholic solutions, have been added. Prior to the application of the jelly-dye mixture, the pollen is washed with 70% ethanol to remove adhering oils and resins. The staining reaction is differential and permits the rapid classification of pollen grains. The "functional" pollen expands and stains with both dyes whereas the aborted grains remain shrunken and take only the methyl green wall stain. The same reaction functions with orchid seed.**

The technic outlined below is a modification of Wodehouse's methyl–green:glycerol–jelly method (1933) and was designed for studies of pollen sterilities and the determination of abortion percentages. One stain, with preference for cytoplasm (e. g. phloxine, rose Bengal), and another for cellulose (e.g. methyl green) are used in combination with the glycerol jelly mountant for rapid preparations of mature pollen grains. The staining procedure is differential and removes all cases of doubt in the classification of the grains. "Functional" grains are fully expanded and stained with both dyes, i.e., the exine and intine are stained green, while the cytoplasm is stained a contrasting red. Aborted grains either remain shrunken and stain only with the green dye, or they may expand in varying degrees depending on the amount of non-autolysed cytoplasm present at the time of mounting; these latter show a mottled, reticulate red stain. Wall structure, as defined by Erdtman (1947), porulation and exine sculpturing are easily distinguishable in all grains. However, due to the masking effect of the cytoplasmic dye, little or no internal differentiation is visible.

The preparation of the glycerol jelly is based on Brandt's formula (Lee, 1946). Forty grams of sheet or granulated gelatin are soaked 2–3 hours in cool water. The excess water is drained off and the hydrated gelatin heated in a water bath until melted. Sixty milliliters of glycerol and 1–2 grams of phenol crystals are stirred into the

[1]This work was completed at the New York Botanical Garden in connection with studies of Petunia (Stout, 1952).

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gelatin and the mixture is filtered through a pad of glass wool. Larger quantities may be prepared if desired since it keeps well.

The keeping qualities of the jelly-dye mixture vary according to the usage (maximum 4–6 months), hence it should be prepared in amounts suited to the anticipated needs. A formula for 50 ml. is given:

1. Two and one-half milliliters of a saturated solution of methyl green (National Aniline, Cert. No. NG 27, was used) in 50% ethanol are added to 50 ml. of the hot melted jelly. A color similar to green ink is produced.

2. Two milliliters of phloxine (National Aniline, Cert. No. NPh 7, was used) or rose Bengal (Coleman and Bell), also in a saturated 50% alcoholic solution are added. The color changes to a deep wine.

The depth of the colors may be used as indicators of the dye concentration because the proportions used are flexible and depend on the reaction of the material and the end results desired. The depth of the final color is best ascertained by placing a drop of the jelly-dye mixture on a slide and adding a cover slip. For rapid approximation, the glass container, in which the mixture is prepared, may be placed over a light source while the dye solutions are added.

Since methyl green deteriorates, the dyes should be added to each new batch of jelly just before it is put into service. Also, reheating causes the jelly to deteriorate, hence it should be stored in convenient sized lots in glass containers. Thus one avoids spoiling a large amount by repeated melting. A fresh container should be started as soon as the contents of the one in use fails to solidify satisfactorily.

The schedule for mounting is as follows:

1. A small amount of pollen is placed on the center of a clean slide. One or more anthers may be gently tapped against the slide to dislodge the pollen or it may be washed out with small drops of 70% ethanol until a sufficient amount is on the slide. If the pollen has been collected in vials, a sufficient amount to cover the tip of a tooth pick is used.

2. The pollen is then washed with 70% alcohol to remove any adhering oils and resins. The alcohol will spread out and evaporate leaving a ring of sludge which is removed by means of a cotton swab moistened with alcohol. This process should be repeated as many times as is necessary.

3. A drop of hot stained glycerol jelly is placed on the pollen mass just before the last drop of alcohol evaporates completely. The mixture is stirred gently with a needle to assure even distribution. The size of the drop depends on the material used and should be large enough to reach the edges of the cover slip (# 1 or 0) when the pressure is being taken up by the grains. The jelly is kept fluid by passing the slide over a spirit flame, heating just enough to sting but not burn the knuckle. Excessive heating will rupture many of the

pollen grains. After the slide has been removed from the source of heat, a clean flamed cover slip is placed on the preparation.

4. The glycerol jelly is allowed to cool and the preparation is stored in a cool place. The cover slip may be sealed with paraffin or gum mastic. The intensity of the cytoplasmic stain increases on standing whereas the action of the methyl green is practically instantaneous. Preparations retain their brilliance for about a year, after which the methyl green bleaches out rapidly.

This technic works equally well with pollen which has been stored dry for a short while. It can be used also for the evaluation of orchid seeds which contain a naked embryo surrounded by integuments.

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CRYSTAL VIOLET AS A NUCLEAR STAIN FOR GAFFKYA TETRAGENA AND OTHER BACTERIA

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Received for publication Feb. 19, 1952

ABSTRACT.—**A method is given to stain the nucleus of *Gaffkya tetragena* and other bacteria. Only water solutions of crystal violet, mercuric chloride, and nigrosin are used. While the application of heat (50°C. for 20 seconds) is not absolutely necessary, it facilitates the decolorizing process. Some of the nuclei of cells from old cultures were found in various stages of division. The nucleus of a mature cell divides, and each daughter nucleus undergoes a second division usually in a plane at right angles to that of the first division. The nuclear division indicates cell development in units of four cells each. The method was found reliable for demonstrating the nucleus at various periods and stages of growth.**

According to Bisset (1950) there are three main problems to be solved in the demonstration of the true morphology of bacteria. They are stated as follows: "Distortion due to drying must be avoided, the masking effect of the strongly staining protoplasm and cell membranes overcome, and those structures demonstrated which, like the cell wall, are difficult to stain." Theoretically, any treatment of cells in the study of the morphology of bacteria is objectionable; but since some treatment is essential, particularly in the study of internal structures, it should be kept to a minimum. It is the purpose of this paper to give a new method for demonstrating the nucleus of *Gaffkya tetragena* and other bacteria which contributes to the solution of the above problems.

MATERIALS AND PREPARATION

The stain consists of a 1% aqueous solution of crystal violet adjusted to a pH of 6.0 to 8.0 with potassium hydroxide. In practice stains of lower pH value were used on cells from young cultures while stains of higher pH values were used on cells from old cultures. For the latter purpose the aqueous solution of stain may be made up to a pH of 7.5–8.0 with aniline or pyridine. These stains apparently penetrate more readily. The pyridine complex is probably the better. Lower concentrations of the dye solution can be used. Other stains, such as methylene blue, basic fuchsin, and methylene green, have been tried, but preliminary work indicates that crystal violet gives the best results.

A 1% solution of mercuric chloride in water constitutes the

mordant. A weaker solution can be used by adjusting the time the cells are being treated.

Nigrosin is used as the decolorizing agent. A 2–5% solution in water adjusted to a pH range of 3.0 to 4.0 with hydrochloric acid is satisfactory. Other concentrations may be used if desirable.

The bacteria were grown upon proteose-peptone–#3-glucose–yeast-extract agar which contained 0.5% each of glucose and yeast extract. Insofar as is known, any medium favorable to the growth of the organism may be used. The cultures were incubated at 30°C. No special treatment is necessary in the cleaning of slides.

Procedure and Results

The cells of *Gaffkya tetragena* of any age are smeared in water on a slide. The smear is allowed to become air dry and then covered with the stain for from 1 to 5 minutes depending upon the age of the culture from which the cells were removed. Cells from young cultures stain more readily and deeply than those from old cultures and also require less time in mordanting. The excess stain is removed from the smear by flooding with water and shaking vigorously. The wet slide is dipped immediately into the mordant for 3 to 20 seconds. A more dilute solution of the mordant (0.1%) may be used in case the reaction is too rapid. The mordant is washed off with water, and the slide allowed to dry thoroughly. A period of 5 to 10 minutes is allowed to pass after the slide is apparently dry. Then the smear is covered with a very thin film of the nigrosin solution by means of a one millimeter loop or by placing a small drop of nigrosin on the slide near the smear and spreading with another slide as in making a blood smear. Although the decolorizing can be done without heat, it has been found desirable to place the slide on a 45 to 50°C. slide-warmer for 15 to 30 seconds before applying the nigrosin. In this case, the film dries almost instantaneously, and upon cooling, it is

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PLATE 1

Fig. 1. — Cells from an old culture. Note cell division. Stained by the reduction-oxidation method. 3000 ×

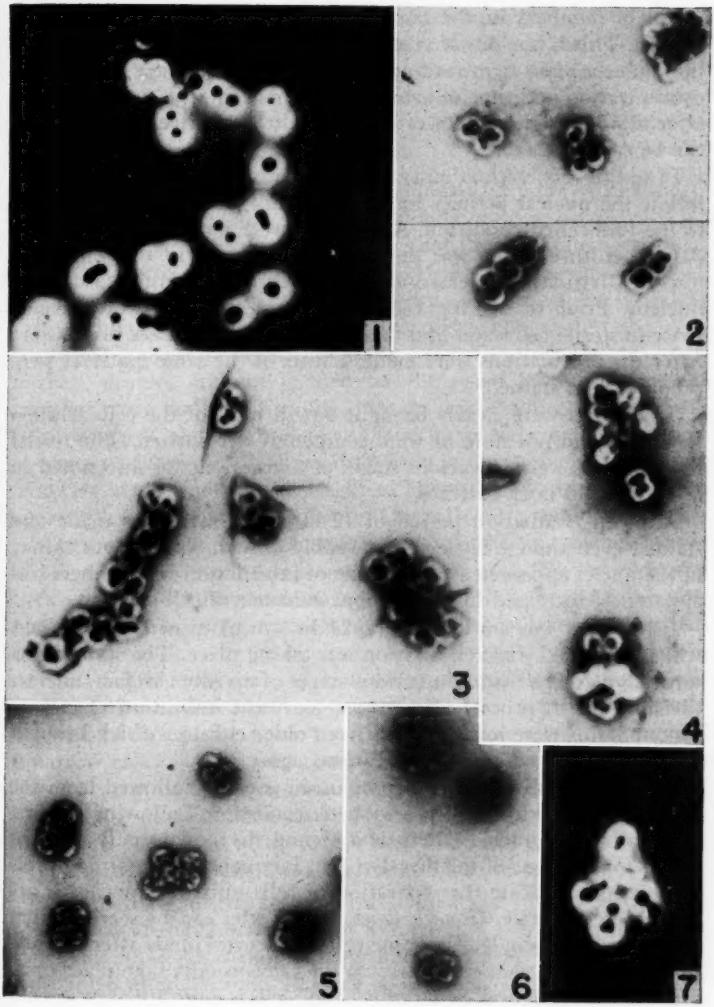
Fig. 2. — Cells from old culture. Note cells with discrete nuclei and nuclei undergoing division. Stained by method reported in text. 2250 ×

Fig. 3 and 4. — Cells from a 24-hour culture. Transfer made from old culture. Note nuclei in various stages of division. Stained by method reported in text. 2250 ×

Fig. 5 and 6. — Cells from a 48-hour culture. Method of staining given in text. 2250 ×

Fig. 7. — A quartet of cells, showing the nuclei of three cells dividing. Stained by reduction-oxidation method. 3000 ×

The writer is indebted to Dr. Leon A. Snyder and Dr. Norman H. Boke of the Department of Plant Sciences of the University of Oklahoma for the photographic work.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ready for observation. It should be emphasized that there are many variables involved in the staining process and it is difficult to be specific. This is obvious after considering the concentration, the pH, the time the stain requires to act upon the cells, the age of cells, the concentration and time of action of the mordant, the time of drying after mordanting, etc. However, with a little practice the procedure can be easily mastered.

In testing the method and technics, it was desirable to attempt to follow the nuclear activity in the subject organism from the single to the four-celled condition. Agar slants were inoculated from a 21-day-old culture which was thought to be mature in the sense that nuclear divisions had ceased and that each cell contained a discrete nucleus. Prior to making the inoculation, the cells were washed twice in sterile tap water and then made up into a heavy suspension. After the inoculations were made, smears of the same material were prepared and stained.

The nuclei were clearly brought out in most of the cells while a few either did not stain or were completely decolorized. The nuclei in some cells were in various stages of division as was also noted in cells from old stock cultures.

After an incubation period of 12 hours, a smear was made and stained even though little if any visible growth was evident. Many of the nuclei appeared as at the time of inoculation while others had apparently increased in size without evidence of cell division.

Incubation was continued (12–24 hr.) until growth was visible, which indicated that cell division was taking place. The stained material showed the nuclei in various stages of division. In fact, nuclear division was in process prior to any outward indication of cell division. Stains were made of cells from older cultures which brought out the nuclear activity at the various ages.

The nuclear division in this organism is easily followed from the single cell to the four-celled or quartet condition. Following the first division of the nucleus there is a second division usually at right angles to the plane of the first division. Frequently the staining process does not indicate the separation of cells until the quartet is approaching maturity. In such cases, four nuclei can be seen in what appears to be a single cell. Some cells show cytokinesis after the first nuclear division though separation does not usually take place until after tetrad formation. Separation of the cells is more apparent earlier in an immature quartet when stained by the reduction-oxidation method (Chance, 1950). The stain shows that the second division of the nucleus of one cell of a pair may occasionally be at right angles to that of the other giving a "T" form instead of the quartet; or both nuclei of a pair may divide in the same plane giving a row of four cells. Sometimes only one nucleus of a pair of cells divides making three cells in a group. In following the nuclear activity, it is

apparent that the cells develop in units of four. The stain also clearly showed the initiation of nuclear division in the cells of some quartets before final separation of cells, or at least they remained in the position of the quartet. The application of the method to cytological problems will be reported in a later paper.

Discussion

The photographs show a stained nucleus surrounded by de-stained cytoplasm. They may also suggest a stained organism surrounded by a capsule. The evidence is to the effect that the first statement is the correct one. According to Bergey *et al.* (1948), this species consists of "spheres: 0.6 to 0.8 μ in size, with pseudocapsules (in body fluids) surrounding four of the elements showing typical tetrads." No statement is made in regard to single cells. Although the inference is that capsules are not formed on the usual laboratory media, transfers were made to lithium chloride agar hoping to break the culture into R and S forms. No change in the type of colonies was observed; neither was there any difference in the stained cells. Transfers were made from the LiCl agar to glucose agar and followed by many transfers upon this medium. The cells from one of these cultures were brought into suspension and washed three times before staining. It would seem that if capsules were present they would have been removed by this treatment. Since there was no observable difference in the staining reaction of the washed cells and those in a single suspension, the washing was not continued. In fact, smears were made from the agar slants and capsular stains (Leifson) made with negative results. The Anthony stain (original formula) also gave negative results. However, in this case, there were a few cells having a narrow clear area around them. These isolated cases could probably be accounted for on the basis of surface tension. The area around the nuclear material (cytoplasm) has also been stained a different color giving a two-tone effect. The stain and method used were different from any known capsular stain or method.

A comparison in size, between the stained and stained-decolorized cells, was made by de-staining only a fraction of a smear. Little if any difference in size was detected by this technic. If the cells are not mordanted after staining, they can be completely decolorized by the nigrosin.

Finally the structures were stained by a dye which has long been known as a nuclear and chromatin stain. The dyes used in the preliminary work were also nuclear stains. The structures stained were in the position of a nucleus, at least in known plant material, and the behavior is that which one would expect a nucleus to have.

Summary

A new method for staining the nucleus of *G. tetragena* and other bacteria involves the use of crystal violet, mercuric chloride, and nigrosin. The method is simple and direct, avoiding many of the criticisms of technics of the past. The nuclei of some cells from old cultures are shown to be in various stages of division. The indications are that the cells of this species develop in units of four or quartets. The stain clearly shows a sequential development in that the nucleus of a cell divides and then each of the two daughter nuclei divides in a plane usually at right angles to that of the first division. In many cases, no wall is observable up to this time, but as the tetrad matures, each nucleus is found in a single cell. The staining procedure was found dependable for demonstrating the nucleus of the test organism at any age.

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HISTOCHEMICAL USE OF SODIUM BISMUTHATE

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Received for publication April 18, 1952

ABSTRACT.—**Histochemical 1,2-glycol cleavage, similar to that obtained with periodic acid and lead tetraacetate, may be obtained with sodium bismuthate. Routinely prepared slide sections, from tissues fixed in 10% formalin, are run down through xylene and graded alcohols to water and then oxidized for three minutes in a 1% sodium bismuthate 20% aqueous phosphoric acid solution. The oxidizing solution must be freshly prepared and used immediately. Following oxidation, sections are rinsed 15 sec. in 1*N* HCl to remove bismuth pentoxide precipitate, a by-product of the reaction. The sections are then washed in distilled water and placed in leucofushsin for 10 min., or in a saturated 30% alcoholic solution of p-nitrophenylhydrazine for 5 min. or 2,4-dinitrophenylhydrazine for 30 minutes. After staining, the sections are rinsed in 30% alcohol if the nitrophenylhydrazines were used, or in the standard dilute sulfite bath followed by running tap water for 5 min. if leucofuchsin were used. Sections are routinely dehydrated, cleared, and covered. On examination, the sites of 1,2-glycol linkages will be stained violet by leucofushsin or yellow by the nitrophenylhydrazines.**

In 1949 Rigby demonstrated that sodium bismuthate, acting in an acid medium, was capable of cleaving the 1,2-glycol linkage in much the same manner as periodic acid (Malaprade, 1934) and lead tetraacetate (Criegee, 1930). In light of the interest which has been shown in the latter two compounds (Lillie, 1950, 1951; Crippa, 1951; Shimizu and Kumamoto, 1952; Lhotka, 1952), sodium bismuthate was investigated as a histochemical reagent.

Sodium bismuthate is superior chemically to both periodic acid and lead tetraacetate. In dry form, it is quite stable and may be stored indefinitely before use. Its oxidations proceed well at room temperature in either aqueous or organic media. Impairment of reaction specificity by side reactions and over oxidation, possible with the other two reagents (Adams et al., 1944; Rigby, 1950; Fuson, 1950) is negligible with sodium bismuthate. Finally, like lead tetraacetate, it not only has the ability to oxidize 1,2-glycols, but also is able to reduce α-hydoxy acids to lower aldehydes (Rigby, 1950).

Sodium bismuthate is a powerful oxidizing reagent apparently acting through acid hydrolysis to reduce the quinquivalent bismuth to a lower order of valency in a manner analogous to the reduction

of quadrivalent lead (Thorne and Ward, 1939; Rigby, 1950). In glycol cleavage, it is believed that the glycol and the bismuthate form a cyclic compound which subsequently splits to form two carbonyl compounds and bismuth pentoxide (Rigby, 1950). Although this reaction may take place in either aqueous or organic media, histochemically, the medium of choice appears to be 20% aqueous phosphoric acid. Using a 1% concentration of sodium bismuthate, a smooth, complete glycol cleavage is obtained in three to five minutes. When the bismuthate and phosphoric acid are combined, a bright yellow-orange suspension is produced. This suspension is replaced by a dark brown precipitate of bismuth pentoxide as the reaction progresses to completion. The precipitate is adherent to the tissue sections, but it is readily dissolved by dilute hydrochloric acid and the histological details are not obscured after staining. To avoid loss of oxidative potential, the bismuthate-phosphoric-acid solution

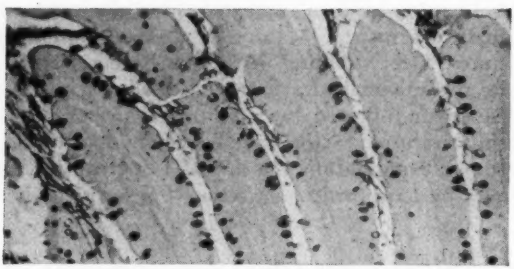

FIG. 1. — Rabbit ileum × 100. Sodium bismuthate, leucofuchsin stain.

must be used immediately after mixing. Otherwise the glycol cleavage will be incomplete.

Several technics may be used to demonstrate the aldehyde radicals released by the sodium bismuthate oxidation. Schiff's reagent gives excellent results, coloring the unmasked radicals violet, or nitrophenylhydrazines may be used to stain the carbonyl sites yellow.

The recommended technic for formalin fixed, routinely prepared, paraffin sections is as follows:

1. Pass mounted paraffin sections through xylene and graded alcohols to water.
2. Oxidize sections for 3 min. in 1.0% sodium bismuthate in 20% aqueous phosphoric acid. The solution must be used *immediately* after mixing the bismuthate and phosphoric acid.
3. Wash in running tap water 1 min.
4. Rinse for 15 sec. in 1*N* HCl to remove adherent bismuth pentoxide from sections.
5. Rinse in distilled water.

6. Stain for 10 min. in Schiff's reagent.
7. Rinse sections in three successive two-minute changes of sulfurous acid.
8. Wash in running water for 5 min.
9. Counterstain (optional) for 30 sec. in Harris hematoxylin. Wash 5 additional min. in running water.
10. Dehydrate through graded alcohols, clear in xylene, and mount in clarite or balsam.

Either 2,4-dinitrophenylhydrazine or p-nitrophenylhydrazine (Johnson et al, 1946; Albert and Leblond, 1946) in a saturated 30% alcoholic solution may be substituted for the leucofuchsin in Step 6. Sections are stained in the former solution for 30 minutes, or the latter for 3 to 5 minutes, and then rinsed in 30% alcohol to remove excess reagent. Steps 7 and 8 are omitted. These reagents should be freshly prepared at the time of staining for best results.

The histochemical picture obtained with sodium bismuthate oxidation is similar to that obtained with both periodic acid and lead tetraacetate in that the tissue sites of compounds containing the 1,2-glycol linkage are stained. Figure 1 shows typical staining of the 1,2-glycol type, in rabbit ileum after sodium bismuthate oxidation, with good demonstration of goblet cell mucin and intestinal cuticle. Investigations are now in progress to note any staining differences between this technic and the periodic acid and lead tetraacetate technics. If findings warrant it, a report will be made at a later date.

Acknowledgement

The author would like to thank Dr. Max Huffman, of this institution, for suggestions given during the course of this investigation.

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DIFFERENTIAL STAINING OF GASTRIC GLANDULAR CELLS

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Received for publication Dec. 26, 1951

ABSTRACT.—**Fundus of stomach is fixed in 10% formalin (aqueous), Bouin's fluid or 5% trichloracetic acid (aqueous). It is embedded in paraffin, and 7μ sections are cut, mounted, deparaffinized and passed to 70% alcohol and then stained as follows: Mordant 3 min. in saturated Bismarck brown in 70% alcohol. Rinse in 70% alcohol, pass to distilled water, then overstain (2 hr.) in aniline blue, 0.5% solution in 2.5% acetic acid (aqueous). Precipitate the anilin blue with 0.5 ml. of 0.1% methyl violet solution (aqueous) dropped on the slide. Leave on 2 min. or less. Wash and differentiate in 70% alcohol. (Parietal cells dark blue). Stain 30 min. in a mixture of hematein, 0.10g.; $AlCl_3$ cryst., 0.05g.; and 70% alcohol 50 ml., prepared just before use and not filtered. Rinse in 70% alcohol and differentiate with an alcoholic extract of saffron (2 g. saffron pistils in 100 ml. 90% alcohol at 60°C. for 6 hr.) while observing the progress of differentiation microscopically. Dehydrate by dropping a 0.1% solution of acetic acid in absolute alcohol on the section for 30 sec., followed by pure absolute alcohol, xylene, and covering in balsam.**

INTRODUCTION

An early method for differentiating parietal and chief cells of the stomach was published by Kolster (1895). The tissues were stained by hematoxylin and then counterstained by acid fuchsin, eosin or Congo red. Later, Harvey (1907) and Kirk (1910) investigated fixation and staining of gastric mucosa. Fixation with Bensley's fluid (3% aqueous potassium dichromate and a saturated solution of mercuric chloride in alcohol, equal parts) and staining by gentian violet, Mayer's mucihematein, or Bensley's acid-fuchsin:orange-G:toluidine-blue stain were recommended.

Our procedure was developed to give a polychromatic stain which would follow formalin, Bouin's or trichloracetic acid fixation, and which possessed a high degree of reliability. The stain is not subject to fading, as was shown by its survival of a test of 48 hours under strong ultraviolet light and by exposure for 4 months on the workbench.

Materials and Procedure

Our research has been performed on sections of stomach from dog, rat, and pig. The fixation of preference was either formalin, 10% aqueous, Bouin's fluid, or 5% aqueous trichloracetic acid. Staining in the block by Hollande's carmine before embedding in paraffin was optional. Sections 7μ thick were cut and mounted, the paraffin removed by toluene, and slides passed through graded alcohol to 70% in the usual manner.

Dyes were from the following sources: hematein, 220195, Ciba, Basel; aniline blue, W. S., 3535, George T. Gurr, London, S. W. 6; methyl violet, 11689, Laboratoria Flandria, Ghent; Bismarck brown, George T. Gurr, London, S. W. 6; saffron (obtainable in the U. S. A. from some wholesale drug houses and from a few fancy grocery stores. *Editor*)

Staining was done at about 20°C. and the following technic is recommended:

1. Stain 3 min. in a saturated solution of Bismarck brown in 70% alcohol. This solution acts as a mordant.
2. Rinse in 70% alcohol.
3. Wash out the alcohol in distilled water.
4. Overstain for 2 hr. in a saturated solution of anilin blue (Gurr) in 2.5% aqueous acetic acid. If Merck's wasserlöslich (water-soluble) anilin blue is used, a 0.5% solution in the dilute acetic acid suffices.
5. Precipitate the anilin blue by dropping about 0.5 ml. of a 0.1% aqueous solution of methyl violet on the section. Leave on 2 min., *not more.*
6. Wash and differentiate in 70% alcohol. The color of the parietal cells becomes dark blue.
7. Stain 30 min. in a solution of hematein 0.1g.; $AlCl_3$ (cryst.) 0.05g.; 70% alcohol, 50 ml. Prepare this solution just before use. Stir from time to time but do not filter. The staining time will require lengthening as the solution ages.
8. Rinse in 70% alcohol.
9. Wash out the excess of anilin blue, and at the same time stain the connective tissue for 3–10 min. in an alcoholic extract of saffron. Prepare this extract by digesting 2 g. saffron pistils in 90% alcohol at 60°C. for 6 hr. Differentiation is complete when the parietal cells appear greenish blue and connective tissue greenish yellow as seen through the golden yellow solution.
10. Wipe the superfluous colorant from around the section and dehydrate and acidulate by dropping a 0.1% solution of acetic acid in absolute alcohol on the section for 30 sec.
11. Complete the dehydration in pure absolute alcohol and clear in xylene or toluene.
12. Mount in balsam or salicylated balsam.

Results. The typical stain gives the following colors: nuclei, violet; peptic cells, brown; parietal cells, blue; muscle fibers, blue; connective tissue, greenish yellow; epithelia, violet blue.

Note. Excessive loss of the saffron stain is avoided by the use of absolute alcohol in Steps 10 and 11. Lower grades are unsatisfactory (Langeron, 1942).

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RAPID STAINING OF BETA CELL GRANULES IN PANCREATIC ISLETS

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Received for publication May 15, 1952

A technic using aldehyde-fuchsin for the demonstration of the beta cells of the islets of Langerhans was first described by Gomori.[1] He recommended that the sections be treated with Lugol's solution for ten minutes to one hour, decolorized with sulfite, washed, and stained with aldehyde-fuchsin for 15 to 30 minutes or longer.

A modification of this technic, which is more rapid and precise, has been developed in a study of the pancreatic islets of the mouse. The material is fixed in Bouin's fluid and stained as follows, all solutions named being aqueous:

1. Bring deparaffinized sections to water.
2. Oxidize in a mixture of equal parts of 0.5% potassium permanganate and 0.5% sulfuric acid for 2 minutes.
3. Rinse in distilled water.
4. Decolorize in 2% sodium bisulphite.
5. Wash under tap for 2 minutes.
6. Stain in Gomori's aldehyde-fuchsin for 2 minutes.
7. Rinse in 3 changes of 95% alcohol.
8. Absolute alcohol, xylene, and mount.

Examination of islets stained by the above procedure discloses that the beta cell granules are stained a deep purple and stand out clearly against the colorless cytoplasmic background. Nothing else in the islets is stained. By this method the different degrees of granulation of beta cells can be very precisely determined and, thereby, estimates made as to the functional states of the cells examined.

If a counterstain is desired, various mixtures may be used, e.g. the light-green–orange-G mixture used by Halmi[2] as a counterstain to aldehyde-fuchsin in differential staining of the anterior pituitary. However, the author has found in the present work that counterstaining with phloxine and fast green FCF has proven most satisfactory.

From step 7 of the procedure outlined above proceed as follows:

1. Run sections down to water.

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[1]Gomori, G. 1950. Aldehyde-fuchsin: a new stain for elastic tissue. Amer. J. Clin. Path., **20**, 665–6.

[2]Halmi, N. S. 1952. Differentiation of two types of basophils in the adenohypophysis of the rat and mouse. Stain Techn., **27**, 61–4.

2. Stain in 0.5% phloxine for 2 minutes.[3]
3. Rinse in distilled water.
4. Place in 5% phosphotungstic acid for 1 minute.
5. Wash under tap for 2 to 5 minutes.
6. Stain in 0.2% fast green FCF for 30 seconds.[3]
7. Rinse in 95% alcohol for 15 seconds.
8. Place in absolute alcohol for 30 seconds.
9. Xylene (2 changes) and mount.

After counterstaining by this method, the cytoplasmic background of the beta cells is stained light green in strong contrast to the deep purple granulation. The cytoplasm of the other cells in the islet and of any duct cells present is also stained light green, while nuclei and erythrocytes exhibit the light red coloration of the phloxine.

[3]Dyes used by the author were as follows: Basic fuchsin, Hartman-Leddon, C. I. No. 677–314; Phloxine, Coleman & Bell; Fast green FCF, National Aniline, Cert. No. NGF – 4.

PINACYANOL ERYTHROSINATE AS A STAIN FOR MAST CELLS

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Received for publication May 8, 1952

ABSTRACT.—**An alcoholic solution of the compound dye, pinacyanol erythrosinate when diluted to the optimum dissociation point is a differential tissue stain which, in addition, selectively stains and differentiates mast cells. It can be made up and used like any other compound dye (*e.g.*, Bowie's stain, neutral gentian, etc. or like a blood stain). It can be used after any of the common fixatives and has the advantage of selectively staining all types of mast cells in their various functional phases, even in those species (notably rabbit and man) in which they may be difficult to demonstrate with other mast cell stains after aqueous fixatives.**

HISTORICAL REVIEW

Many of the dyes employed in histological stains are photographic sensitizers. (Mees, 1942). As early as 1883 Eder had found that erythrosin (tetraiodo-fluorescein) was a superior green sensitizer and it has been used since 1884 in making orthochromatic plates. In 1905 Homolka produced a new red sensitizer from the cyanine group of dyes which was named pinacyanol. In 1920, Mills and Hamer showed that it belonged to the class which they termed carbocyanines. Plates, sensitized with pinacyanol (1,1′-diethyl–2,2′-carbocyanine iodide) combined with pinachrome, were produced commercially as panchromatic plates.

In 1930, R. R. and R. D. Bensley made a neutral salt of pinacyanol and erythrosin in order to pan-sensitize photographic plates by the bath method. At this time they found that this salt was also a differential histological stain. Moreover, mast cells were seen where they had not been seen before. Since then we have attempted to determine the factors which influence staining with this compound dye, to standardize the technic, and to test its selectivity for mast cells.

In the mean time the use of pinacyanol alone (Conn, 1946, Cowdry, 1948), as a histological stain, has been described by others. Proescher (1933) used a dilute alcoholic solution of pinacyanol for staining frozen sections and demonstrated the metachromatic properties of the dye. Hetherington (1936) used pinacyanol as a supravital stain for mitochondria in blood cells. But the use of this dye in histological staining has not been fully exploited.

With the growing interest in mast cells, it may be appropriate

at this time to record our experience with the use of pinacyanol erythrosinate as a selective stain for these cells. (Bensley, 1948, 1950).

Method

FIXATION

Tissues fixed with 10–15% formalin in water, saline or alcohol, 95% or absolute alcohol alone, Bouin's, Zenker's, Helly's and Regaud's fluids have been stained successfully.

MATERIAL

Various organs and tissues from the following species have been studied with this method: mouse, rat, guinea pig, rabbit, cat, dog, monkey and man (biopsy and autopsy material).

TECHNIC

To make up the neutral dye:

Dissolve 100 mg. of pinacyanol (Eastman Kodak Company, #622) in 100 ml. distilled water in a large Erlenmeyer flask. This takes about 24 hours. Keep the flask corked, in a cool dark place, and agitate the solution occasionally. Do not let it stand more than 24 hours, as the dye changes and starts to precipitate.

Dissolve 100 mg. of erythrosin (National Aniline and Chemical Co. C. I. 773, dye content 87%, cert. No. 6) in 100 ml. distilled water. It goes into solution rapidly.

Add about 75 ml. of the erythrosin solution to the pinacyanol solution while agitating the latter. A fine precipitate should begin to appear on the side of the flask.

To complete the precipitation add more erythrosin solution, a few drops at a time, shaking the flask between each addition. With each addition, test for complete precipitation in the following way:

With a stirring rod, remove a drop of the mixture and put it on a filter paper. Around the small amount of precipitate there will be a colored ring. When the ring is just faintly pink, the precipitation is complete. If too much erythrosin is added the dye is unsatisfactory and might just as well be discarded. If the 100 ml. of erythrosin is not sufficient to complete the precipitation, a small amount of erythrosin can be put into solution and carefully added to the mixture.

When the precipitation is complete, filter the mixture through filter paper, or centrifuge it. If filtered, do not wash the precipitate. Remove the filter paper and dry it and the precipitate completely in a low temperature incubator (not above 37°C.). If centrifuged, decant the supernatant fluid carefully and dry the sediment completely.

When dry, dissolve the precipitate in about 150 ml. of absolute ethanol (or methanol) and store in a tightly stoppered dark brown

bottle in a cool place (preferably the refrigerator). Filter paper supporting the dried precipitate may be cut into strips and put into the bottle and the alcohol added. This constitutes the stock solution of pinacyanol erythrosinate (PE). It deteriorates slowly on standing but can be used for about one year.

Staining solution:

To 45 ml. of 30% ethanol or 30% methanol (prepared from the absolute reagents), add 5 ml. of the stock PE solution. Mix well and pour into a Coplin jar, away from heat and direct sunlight. The staining solution should be made up just before the sections are to be stained, and should be made up fresh for each set of 25 slides.

Staining procedure after formalin, alcohol or Bouin's fixatives:

1. Remove paraffin from sections with xylene
2. Absolute alcohol: to remove the xylene
3. 95% alcohol: rinse
4. PE staining solution (in a cool, shaded place) 5 – 30 minutes. (If the sections are not stained in this time the stain is unsatisfactory and a fresh staining solution should be made).
5. Blot slides between filter papers.
6. Rinse rapidly through absolute alcohol and xylene and examine the section with the microscope. If the section is too intensely stained it may be carried back through the absolute alcohol to 95% alcohol to be further differentiated, and then back through the absolute alcohol and xylene.
7. Second change of xylene (or into benzene) 10–30 minutes.
8. Mount in balsam and cover with a cover slip.

Staining procedure after fixatives containing mercury and chromates:

1. Xylene
2. Absolute alcohol
3. 95% alcohol
*4. 70% alcohol with I_2 until all mercury crystals are removed.
5. Wash thoroughly in 1–5% sodium thiosulfate ("hypo") to remove all iodine.

   Traces of iodine in the section produce a precipitate of needle-like crystals from the dye, and interfere with staining. *Steps 4 and 5 are necessary only when fixatives containing mercury have been used.
6. Wash thoroughly in distilled water.
7. Place in 1% potassium permanganate – a few seconds.
8. Rinse in distilled water.
9. 10% sodium metabisulfite – until the section is white.
10. Rinse in distilled water.
11. Rinse well in 95% alcohol.
12. PE staining solution, as in the preceding directions.

Results

Nuclei – bright blue; cytoplasm of epithelial and muscle cells – sky blue; collagen fibers and ground substance – pink; acidophiles – bright red; mast cells – purple, deep blue, magenta or lavender (depending on fixation and functional state); megakaryocytes – lavender.

Comments

Some of the factors which influence staining with pinacyanol erythrosinate may be mentioned. Pinacyanol is one of the "fugitive" dyes and is sensitive to heat, light and air, especially in aqueous solutions. Therefore in making up the dye and during the staining procedure, exposure to these factors should be avoided as much as possible.

It is important, also, to achieve an accurate precipitation of the salt. This can be done if care is taken to get all of the pinacyanol into solution in the water without letting the solution stand too long, and by avoiding an excess of erythrosin.

The precise staining depends on the proper dissociation of the salt, and the dilution of the alcohol appears to be rather critical in this regard. Too small a dilution of the alcohol results in inadequate dissociation and a muddy staining with the colored salt. On the other hand, too great a dilution results in a precipitation of the pinacyanol and a feeble staining with the erythrosin. For this reason it is better to bring the section from 95% alcohol rather than from water into the staining solution. This in part counteracts the small amount of evaporation of alcohol which may occur during the staining process.

Modifications of the staining procedure may be useful. The stock alcoholic solution of PE may be dropped on the slide and diluted with water as in the method for staining blood films with Wright's, Giemsa, or Leishman's blood stains. Sections stained in the Coplin jar may be more adequately differentiated by employing the clove oil method used for the neutral gentian stain (Cowdry, 1948).

Sections properly stained with PE may gradually fade, especially when exposed to strong light. However, some sections which have been demonstrated for fifteen years are still useful. If rapid fading occurs it is usually due to one of the following factors: 1) hydration before, during or after staining; 2) inadequate dehydration after staining; 3) inadequate clearing; 4) leaving the section too long in the clearing agent; or 5) impurities in the mounting medium.

The staining of mast cells is most unpredictable. Sometimes they stain with hematoxylin and eosin, either blue or red. Sometimes they stain with one or another of a number of anilin dyes. Sometimes their granules are selectively stained and sometimes their cytoplasm stains intensely leaving the granules unstained. Some-

times mast cells remain unstained by dyes which are generally used for staining them selectively. Whether all mast cells are always stained with PE is impossible to determine. Certainly this stain has differentiated more mast cells more consistently in sections of fixed material than any other stain which we have investigated.

When attempting to determine the number and character of the mast cells in any given section it is wise to observe certain precautions even with PE: 1) A section of tissue known to contain mast cells should be stained along with the unknown to test the efficiency of the stain; 2) if possible, adjacent or similar sections should be stained by at least two other methods which are known to stain mast cells. This serves to substantiate the selectivity of the PE stain.

It has not been possible to demonstrate any chemical basis for the staining of mast cells and their granules with pinacyanol. At present we can only say that pinacyanol in this procedure stains them selectively.

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LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

ANIMAL MICROTECHNIC

ADAMSTONE, F. B. **Structure of the Golgi apparatus of spinal ganglion cells as shown by the application of desilvering methods to standard Da Fano preparations.** J. Morph., **90**, 201–11. 1952.

Spinal ganglia from baby pigs were either (a) fixed *in toto* in Da Fano's cobalt nitrate-formalin (see Microtomist's Vade-Mecum, *10th Ed.*) sectioned at 6 μ, and affixed to slides, or (b) frozen immediately, sectioned at 6 to 10 μ, affixed to slides, and fixed in Da Fano's cobalt-nitrate-formalin. In either case the slides were silvered by the standard Da Fano method. Both types were studied and photographed. After this the preparations (1) were soaked in xylene to remove the cover slips, (2) hydrated (100, 95, 85, 70% alcohol), (3) desilvered in alcoholic iodine solution until desired point had been reached, (4) washed in several changes of 70% alcohol, (5) placed in 0.75% silver nitrate to remove free iodine, (6) placed in sodium thiosulphate solution to remove unreduced silver, (7) washed in running water, (8) soaked in distilled water, (9) dehydrated in alcohol series, (10) cleared in xylene, and (11) mounted in balsam. When compared with records of the original slides, these desilvered preparations gave finer detail of the Golgi net, and confirmed the view that it is not an artifact. — *Elbert C. Cole.*

BRYGIDER, WOLODYMYR. **In what embryonic stage do the eggs of *Neodiprion* enter the winter diapause?** Canad. J. Bot., 30, 99–108. 1952.

As part of a research program on the life histories of eastern Canadian Diprionidae, the early embryonic development of *Neodiprion banksianae, N. nanulus,* and *N. sertifer* was studied by the following methods: Fix eggs 16–20 hours without removing from pine needle pockets in Bouin's fluid or Gilson's fluid (formulae of Becker and Roudabush); transfer to 70% alcohol and renew until no further color change is visible; after Gilson's fluid add KI drop by drop to the alcohol to give the color of cognac, remove possible sediments and transfer to pure 70% alcohol, renewing until colorless; embed in paraffin by Peterfi's methylbenzoate-celloidin or Graupner Weissberger's dioxan method; stain with Mayer's acid hemalum or Ehrlich's acid hematoxylin and counterstain with eosin, acid fuchsin, or orange G; Mayer's carmalum with light green gives the best contrast between yolk nuclei and yolk spherules; Heidenhain's iron hematoxylin shows very well the small nuclei of the minute cellules of the embryo but stains both the nuclei and the yolk spherules black; to obtain best results staining must be watched under the microscope. — *H. P. Riley.*

SCHEFF, IRENE M., and SCHEFF, GEORGE J. **Evaluation of the Wright and supravital staining methods in infections with mononuclear cell response.** J. Lab. and Clin. Med., **39**, 772–9. 1952.

Comparative studies with the Wright and the supravital staining methods show a significant difference in results. The neutrophilic count is lower by the Wright technic, possibly because of mechanical injuries to the cells. Monocytes and some intermediate lymphocytes are more sharply defined by the supravital method than by Wright's stain. The supravital method should be much more widely used.
— *John T. Myers.*

SCHWARTZ, H. P., RIGGS, H. E., GLICK, C., CAMERON, W., BEYER, E., JAFFE, B., and TROMBETTA, L. **Infrared spectroscopy of tissues. Effects of insulin shock.** Proc. Soc. Exp. Biol. & Med., **76,** 267–72. 1951.

Nervous tissues as well as other types from normal and insulin shocked rabbits were rapidly frozen and sectioned at 25 and 50 μ. These sections were prepared by the technic of Adamstone and Taylor (Stain Techn., **23,** 109, 1948) and dried at a low temperature on AgCl plates. Normal control sections were also made at this time. The tissues were analyzed by a Beckmann 1R2 infrared spectrophotometer. The waves produced were correlated with the "protein polyamide" vibration to give a spectrum of the slide. These bands were found to be often diagnostic of the tissues, and appeared to be specific for many of the nucleic acid derivatives. Because of the qualitative reproducibility of the tissues' spectra, an effort was made to quantitate them. Tissue thickness was proved to be independent of the quantitative results. The procedure showed a quantitative difference between normal and insulin-shocked nervous tissue. — *Byron A. Myhre.*

HISTOCHEMISTRY

BERLANGER, L. F. **Detection of phosphatase activity in polarized light following glycerophosphate incubation.** Proc. Soc. Exp. Biol. & Med., **77,** 266–8. 1951.

Tissues of rats and hamsters, including demineralized teeth, were embedded in paraffin or celloidin, sectioned at 10 μ, mounted on a glass slide and incubated at 37° C. in Gomori's solution of sodium alpha glycerophosphate or other substrates at pH 5.0 and 9.4 for 3 hours. After this they were mounted in alcohol-ether or 5% celloidin and covered with a cover glass. These were viewed through a polarizing microscope, the location of precipitates observed, and their amounts quantitated by the use of a light absorption procedure similar to that of Dempsey (Anat. Rec., **96,** 221, 1946). A control series was processed by the technic of Gomori (J. Cell. & Comp. Physiol., **17,** 71, 1941). Advantages claimed for the new technic are the elimination of many artifacts due to the non-specific blackening of the cobalt sulfide by the tissues on the slides and also the possible displacement of the precipitates during the subsequent steps. — *Byron A. Myhre.*

HOLT, M. W., and WARREN, S. **Radioautographic solubility studies of I^{131} and P^{32} in frozen-dehydrated tissue.** Proc. Soc. Exp. Biol. & Med., **76,** 4–9. 1951.

Rat thyroids were sectioned and prepared by using the technic of Holt, Cowing, and Warren (Science, **110,** 328, 1941). The tissues were then mounted, exposed to the test solvent and visualized by the use of radioautography. It was found that paraffin and xylene caused little if any scattering of the rays. Little solubility of the I^{131} was shown by chloroform, anhydrous ether, 5% trichloroacetic acid, Bouin's solution, 95% alcohol, acetone, 1% $FeCl_3$, 5 *M* NaCl, saturated ammonium sulfate, and strong mineral acids and bases of approximately 1 *N*. Blurring of the image with appreciable solubility occurred in KI solution. Essentially complete loss of the image occurred with distilled water, 1 *M* NaCl, 30% urea, 1% pepsin solution, and sodium lauryl sulfate. These results were felt to correlate with the theory that the isotope is incorporated in a water soluble protein molecule. The experiments were repeated on rat livers and other tissues with P^{32}. These results were essentially the same except that with saturated Na_2HPO_4 the phosphorus remained intact. A procedure is given to determine the solubilities of radioisotopes in tissue and this is illustrated with 4 photomicrographs.

— *Byron A. Myhre.*